

**Supplemental Material**

**Diethylstilbestrol (DES)-Stimulated Hormonal Toxicity is Mediated by ER $\alpha$  Alteration of Target Gene Methylation Patterns and Epigenetic Modifiers (*DNMT3A*, *MBD2*, and *HDAC2*) in the Mouse Seminal Vesicle**

Yin Li, Katherine J. Hamilton, Anne Y. Lai, Katherine A. Burns, Leping Li, Paul A. Wade, and  
Kenneth S. Korach

**Table of Contents**

Supplemental Material, Table S1.....	2
Supplemental Material, Table S2.....	3
Supplemental Material, Method.....	4
Supplemental Material, Figure S1.....	5
Supplemental Material, Figure S2.....	6
Supplemental Material, Figure S3.....	7
Supplemental Material, Figure S4.....	8
Supplemental Material, Figure S5.....	9
Supplemental Material, Figure S6.....	10

**Supplemental Material, Table S1. Real-time PCR primers.**

<b>Gene symbol and GenBank accession number</b>	<b>Sequence (5'–3')</b>
Mouse <i>Svs4</i> (NM_009300)	F: GGAGCCATATCGCCAGAACA R: TCCAGGGACTGCCTCTGAGA
Mouse <i>Ltf</i> (NM_008522)	F: CAGCAGGATGTGATAGCCACAA R: CACTGATCACACTTGCGCTTCT
Mouse <i>Pgr</i> (NM_008829)	F: GATGAAGCATCTGGCTGTCACT R: TTATGCTGAACTTCCATTGC
Mouse <i>Stat3</i> (NM_011486)	F: CCTCTATCAGCACAACCTTCG R: CCACGATCCGGGCAATT
Mouse <i>Stat5a</i> (NM_001164062)	F: CCGTGGGATGCTATTGA R: GAGTTGGGTGGCCTGAC
Mouse <i>DNMT1</i> (NM_001199431)	F: GGAAGGCTACCTGGCTAAAGTCAAG R: ACTGAAAGGGTGTCACTGTCCGAC
Mouse <i>DNMT3A</i> (NM_001271753)	F: TGGAGAATGGCTGCTGTGTGAC R: CACTCATCCCGTTTCCGTTTG
Mouse <i>DNMT3B</i> (NM_001003960)	F: AGTGACCAGTCCTCAGACACGAAG R: ATCAGAGCCATTCCCATCATCTAC
Mouse <i>MeCP2</i> (NM_001081979)	F: CTTGCTCTAGGGAGTAATC R: TTGCACTCCATTGTCCAGCT
Mouse <i>MBD2</i> (NM_013594)	F: AACTTTCGACTGACCTTCCC R: ACCTTTCAGTAGACTGCAC
Mouse <i>MBD3</i> (NM_010773)	F: CCACACTGAAAGCAGATGAG R: TCCAAACCCTGGCCTGTAAT
Mouse <i>HDAC1</i> (NM_008228)	F: AGGGACCCCTGCAATATCAA R: CAACCTCCACCCTACAGAAT
Mouse <i>HDAC2</i> (NM_008229)	F: GGTCGTAGGAATGTTGCTGA R: AGCCAATGTCCTCAAACAGG

**Supplemental Material, Table S2.** Bisulfite conversion sequencing primers.

<b>Gene symbol and GenBank accession number</b>	<b>Sequence (5' – 3')</b>
Mouse <i>Svs4</i> (NM_009300)	F: TTGTTAGAAAAGATTTTGATTGAAGG R: TCCCTATACCATTTATCAATACAACA
Mouse <i>Ltf</i> (NM_008522)	F: GGTTAATTTTGGGTGGTATTT R: AAACAACACTCAAACCTAACCAC
Mouse <i>Pgr</i> (NM_008829)	F: TTTAGGAGATAGGGGAGGAGAAAAG R: TAACTTCTACCCCAAAAAAACTCC

## **Methods**

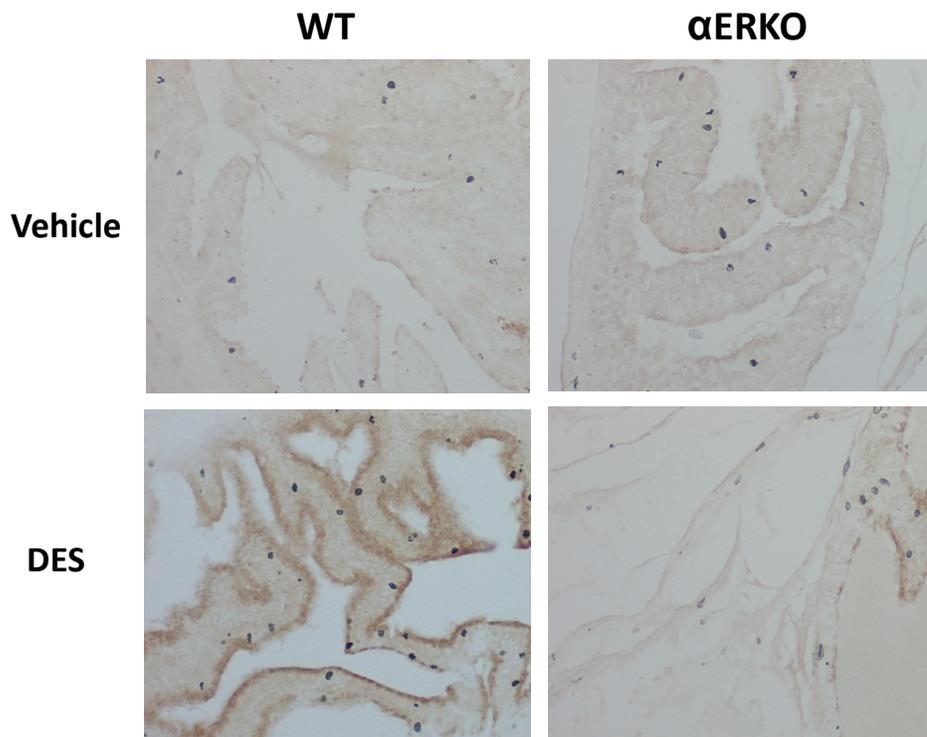
### **Lactoferrin (LF) immunohistochemistry (IHC)**

Mouse SVs were placed immediately into 10-13 ml of Bouin's solution after euthanasia. Tissues were rocked on an Adams Nutator (Clay Adams, Parsippany, NJ) in Bouin's solution for 24-30 h before the Bouin's solution was disposed of and 70% ethanol was added to the tissues. Tissues were processed and embedded in paraffin once the solution remained clear in 70% (w/v) ethanol for several hours. Embedded tissue was sectioned (7  $\mu$ m) and placed onto charged-glass microscope slides for IHC staining. Anti-LF antibody (sc-14434) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Slides were deparaffinized and immunostained according to the manufacturer's instructions with the AEC kit (Zymed Laboratory, South San Francisco, CA) and counterstained with hematoxylin (Sigma, St. Louis, MO).

### **Statistical analysis**

Two-way ANOVA with Bonferroni post-test was performed using GraphPad Prism version 6.00 (\*\*\*,  $p < 0.001$ , Supplemental Material, Figures S4A and S5).

**Supplemental Material, Figure S1.** Immunohistochemistry of LF in SVs of WT and  $\alpha$ ERKO 10-weeks old male mice. Mouse SVs were fixed as described in Supplemental Material, Methods. Slides were deparaffinized, immunostained with anti-LF-antibody, and counterstained with hematoxylin. A strong staining of LF was found in WT DES group samples. (Magnification, 100x)



**Supplemental Material, Figure S2.** The 2 kb sequence surrounding the transcription start site (+/- 1 kb) of the *Svs4* gene. The underlined sequence is the upstream 1 kb promoter sequence as the gene is transcribed from the minus strand. The black bold ACT is the transcription start site (TSS). Teal highlights represent the CpG positions tested in this study.

CpG positions are -160, -237, -306, and -367 bps upstream of TSS.

TATTTCTGGAATTC is putative Stat5a/5b site at -132/-146 bp upstream of TSS.

The red sequence is a predicted Sp1 site (+118/+128 bp).

The blue sequence is a predicted ERE.

**>mm10 *Svs4* chr2: 164277307-164279307**

TCTGACAGTTTCTTCCGACTGCAAGAATTTTTCTGATGAGAGAAAAACAGGCTTTGCTCTCTAC  
CTGTATATTTCCATATAGTCTGGGATTGTTATAAACCAAGACAAGGCAAACAACCTTTGCTTTG  
ATTAGTAACCTATCTTGTGATGCAATTTGCCATTTTTGTTGTCTAGAAATTCCTTGATCTTTTGT  
CTAGTCAGAAATAGGCTTAATTGTGAGCGCCTGACTCACCAGCAAAGCAGACACCACAACAG  
GATCCTTCTACAACATGCCCCGAGCCAGAACTGGTGCTGCTTATATAGGCCTAGGAGAGGCG  
TGTCTCACACCTGGATTGGTTATGCATTCTACCTCATTGTCATGTCTCTCATCTGATTGGTTATT  
CTCTCTCTCTCAGTACCTCACTGAGCCTCATTATCATACTCATTGTCATGTCTCTCATCTGATT  
GGTGATTCTCTCTCAGTATCTCATGGAGCCTCATTATCATGCCTAGGCCAGGCAGTGACTTGGT  
GAAAACTTTACTGCATATGTACACATTGGTTGTTTACCCAGACTTATGCGAGGTGGCCAGCG  
GTAGCCAGCGCCACCTTGTAATGGCATAATGTGGCTTCCCACACTTAATCTGTTTGGAACTGCC  
CTTGATACACTCTCCTTATAATGAACAATAAGAATCTTTAAGTTACATGACCTCCAAGTGTCC  
CTTGCACTTACATCTCTGATTCTTGGTTTATCAATTCAGTGATACCCACACAAAAGCTATGGT  
TACAGCTAGCAAAGACTAACTTCTCATCAGAAATTAGAGTCTCTAGAAAGTAACGCTCTTTAG  
TTCCTCAGAGTACCCTTTAAAGAACCCTGTCAATTGTTTCTTCTCCCCCCCCCATCAACTCTT  
CCTACTCACCTTAGTTTTCTTCCCAATGGCTCCTGTCAACCAGAAAGGAGAAGCAGAGAAAAGA  
GGAACAAACTGGTAGAATTCATCTTGCCAGAAAAGATCTTGACTGAAGGCTAAGCTGAGTTCT  
TAATTTATATATTTCTCAGGTGGGTGTCCCTGGAGTTATAAAAGGCACACCCCACCTTACTTAT  
CTGTAAATTCTGGCTTCTTCCCCTGTGCTATGCAGGGGGTATTGTCATTATTTCTGGAATTC  
ATAAAAGCACATCGTATCAGAGACTTTCTTTTATACCTCCAACTCCAGCCAATCTTTATCAAC  
ACTTGAGATCATTTCACACAGTTTCCGGCATTTCTGAAAGACATTCAGTTGGGACTGAAGAAG  
ACTGCCCTTCATGGTACAAAGTGACTATCCCGTAATCAATATTTCCACAGCACAAGTGTATGT  
CTCTTTCATATACATCCTATAACTTTGCGCTTATGATTCTCTCAAATAAAACAAATATGTTGTACT  
GACAAATGGCACAGGGATAGAAAGACACAGAATGAACTCAAGAAATCCAATGAAAACCTCA  
ATAGGGCAGCTAGAAGAACTCAGGGGAAAGCCTCACCCACAGAATGGGTCATGTCCGGAAT  
TGGAAGGTAGAGGATGGAGAACACATAAAGGCATGGAATCATTCCGGGGAAGCTCAATACGAT  
TTTTTTTTTTTTTGATGCATCAGTGGAAACCACGGGAGAGTTTTGAGACACTGAGAAAAAGATGT  
AACCTCTGAGTTGTGGAGCTAGAGGACAGATGAAAGAACAGCAGAGCAAAGGCATAGAGAAC  
ATCTTCCATAAAAAGCATAAAAAAAGTCAAAACCCTAAAAAAGAGATGTCCATCTTTGCATAAG  
AGGCTCACTGAACACCCAATAGAAAAGACCAGAAAAAACTTCTCTATGGTGTAGTTAAAATC  
TTTAAAACAGATAATAAAGGGTATTTAAAAGTCAAGTACTTATAAAGGCAG  
GTCACCGGAATAACAAATGATAACAATAACAATAACAGGAATCTGTAAAGCCAGGAGGGCC  
AAGAAAGTCCAAGTCTGAAAGATCACAACTGTC

**Supplemental Material, Figure S3.** The upstream 1 kb sequence from the transcription start site (TSS) of the *Ltf* gene. The black bold **CAG** is TSS. Teal highlights represent the CpG positions tested in this study.

CpG positions are -449, -459, -470, -528, -542 bps upstream of TSS.

The red and blue sequences are predicted EREs.

AGGTCANNNTGACCC is a consensus ERE site.

ERE (-324, well-known)

**AGGTCAAGGTAACCC**

||||| | |||

AGGTCANNNTGACCC

Predicted additional EREs

**AGACCAGGCTGGCCT**

|| || |||

AGGTCANNNTGACC

**TGGGGACTGTGACTC**

|| | |||

AGGTCANNNTGACCC

Good half site

**AGGTCACCCAGCACA**

||||| |

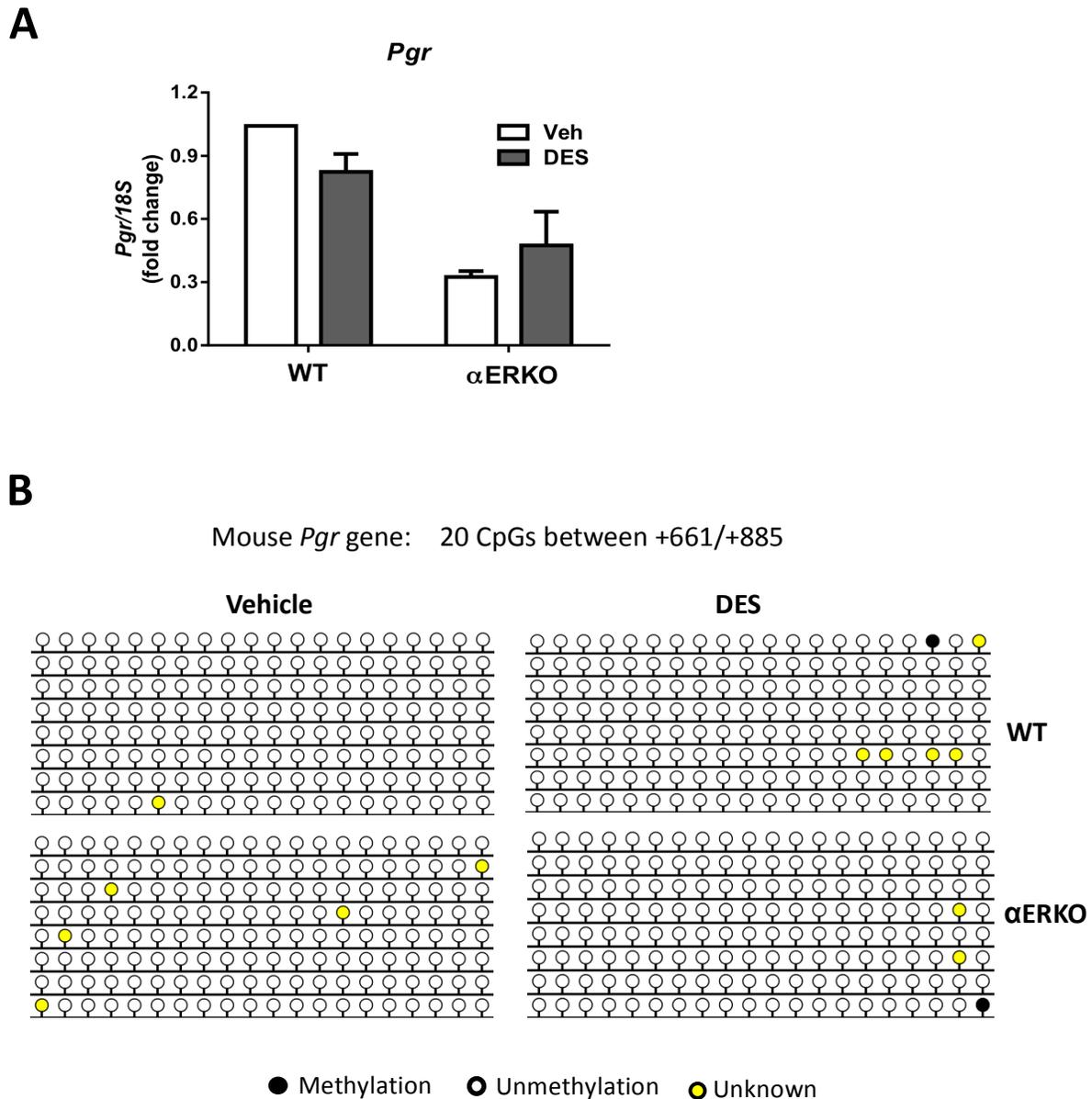
AGGTCANNNTGACCC

The green sequences are Sp1 sites.

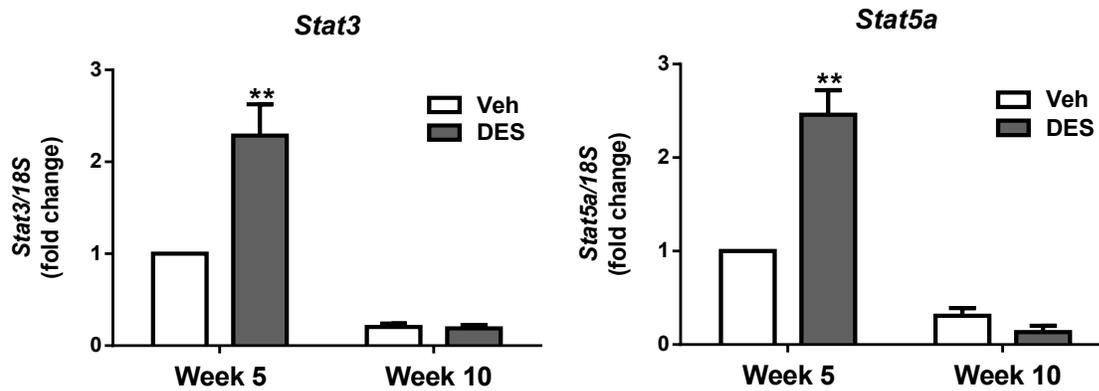
**>mm10 *Ltf* chr9: 111018292-111020291**

CTGGAACCTCACTCTGT**AGACCAGGCTGGCCT**GGAACCTCAGAAATCTGCCTGTCTCTGTCTCCCAAGTG  
 CTGGGATTAAGGCATGCGCCACCACCGCCGGCTCATAATATCATAATATGACTCTTAAGACATTCA  
 TTA AAAAAGAAGTTCTCATTTCTATACCACACATGTACACACACACACACAATCTCTCTCTCACACAC  
 ACATACACACACACACACACACACACACACAGACTCACACACACAAACACACAGACACAAAAATAGA  
 CACGGTACACAGATAGACATAGACACAGACAGACACACAGAGGAAGAGGAAAAAAGATATGGAGGA  
 AGGAATGAAAGAAAAGGAGCAAAAGAAATCAAATGTCAGGGTCTCTCCTCGCTAGCAAATGAAGGG  
 ACACAGGTCAACCCTGGGTGGCATTCTTTCTGAGGTCCTAGGTTATTTT**CG**GGGCTGTATGG**CG**GGT  
 TTCAAGGCAGTGTGGACCCACAGGAACCCTGTGTGCAAGTCTAGGCTGACTC**CG**CTCTCCCTG**CG**GC  
 TGTAC**CG**GGCTGCTGTTGTGGCCAGGCCTGAGCAGCTGCCTCTTCTTTAGAATCCACCACTCTTTGTC  
 TAGCCAAGGAGGAAGGGGATTTGCTTGCTCCATGCAGCTTAAGTGTAC**AGGTCAAGGTAACCC**ACA  
 AATATAG**CCCCCTACCC**ATGTCCACCTCTAGAAAGTACTGGAAACAGAGAAAGGAGAAGACTT  
**GGGGACTGTGACTC**TGATCCTGCAGAAGCTGGGTGGAGATTAAGGAAATCACTCGGTTTCCTGTACC  
 AGCGCCTGTGTAGGGGGTACTGGAGTCCCTGTTTCCTCTTCTGGGCTCCAGGAAGCTGGCCTCTAAGA  
 ACTAGCACACCTGGTTGAGGGCAATGGGGCTGGAAGGCAGGCCTATTGGGCAAT**AGGGTGGGGCCA**  
 GCCCGGT**AGGTCACCCAGCAC**AGATAAAGGGCCCC**GGGAGAGGGC**CAGAAGCCAG

**Figure S4.** Expression and DNA methylation of the *Pgr* gene in week 10 WT or  $\alpha$ ERKO SV. (A) Total RNA samples were extracted from SV tissues of three individual 10-week old WT or  $\alpha$ ERKO mice after neonatal exposure to vehicle or DES. mRNA levels were quantified by real time-PCR. Data shown represent mean fold change ( $\pm$  SE) relative to the WT SV vehicle samples. (B) DNA methylation status of the *Pgr* gene. The region containing twenty CpGs was amplified by PCR from bisulfite-treated genomic DNA, and then the PCR product was subcloned into the pCR-TOPO-XL vector. The sequencing analysis of bisulfite-covered DNA was performed using CpGviewer program. Each line of circles indicates an individual clone sequenced in the analysis after bisulfite treatment and PCR. Open circles indicate un-methylated CpGs. Black circles indicate methylated CpGs. Yellow circles indicate undetectable CpGs (unknown).



**Supplemental Material, Figure S5.** Changes in Signal transducer and activator of transcription (*Stat*) 3 or 5a after neonatal DES exposure in WT SV. Total RNA samples were extracted from SV tissues of individual 5- or 10-week old WT mice after neonatal exposure to vehicle or DES. The expression levels of *Stat3* and *Stat5a* were quantified by real time-PCR. Data shown represent mean fold change ( $\pm$  SE) relative to the WT SV vehicle samples at week 5, \*\* $p < 0.01$ .



**Supplemental Material, Figure S6.** Working model. DES-induced toxicity is mediated partially by ER $\alpha$ ; three epigenetic markers are involved in the epigenetic programming during neonatal DES exposure. For DNA methylation analysis, the 4 CpGs in the *Svs4* gene promoter changed from un-methylated to methylated and 2 CpGs in the *Ltf* gene promoter changed from methylated to un-methylated. These alterations of DNA methylation associate with the expression level of the genes.

